CHAPTER 3

MATERIALS AND METHODS

3.1 Raw material

3.1.1 Negative honey sample

The honey samples to be analysed in this study was longan honey that was produced by *Apis mellifera* in standard bee farm which practised a good beekeeping system. The honey was collected during February-April 2009 from Chiang Mai, northern part of Thailand. Honey was examined for the physical property (color quality using a colorimeter (Minolta, CR-410, USA.)), the chemical properties (pH using a pH meter (Cyberscan, model 310, Singapore)), total soluble solids using a hand refractometer (ATAGO, model N-3E, Japan), water activity (a_w) using a water activity analyzer (AQUA LAB, model Series 3TE, USA.), hydrogen peroxide (Quantofix®, Peroxid 25, Germany), tetracycline group residues by HPLC techniques and the microbiological properties (yeast and molds by ISO 21527-2 (2008), total bacteria count by ISO 4833 (1991)). Blank honey was stored at 4-8^{o}C in a refrigerator until processing. This honey sample was aseptically collected in sterile screwed caps.

3.1.2 Commercial Honey samples

Honey samples were selected randomly from different geographical origins and private beekeepers in the north of Thailand (76 samples constisting of Longan (51), Bitter bush (11), Wild flower (7), Sunflower (5), Lychee (1), Para rubber (1). The 44 samples (Unknown (16), Longan (14), Wild flower (5), Sunflower (2), Rambutan (1), Sesame (1), Acacia (1), Orange (1), Eucalyptus (1), Macadamia (1), Lychee (1)) were purchased from local markets or supermarkets in Chiang Mai, northern part of Thailand during June-August 2010.

All honey (120 samples) were stored at room temperature in darkness until testing. All of them were from multi flower origin (Longan (65), Bitter bush (11), Wild flower (12), Sunflower (7), Lychee (2), Rambutan (1), Sesame (1), Para rubber (1), Acacia (1), Orange (1), Eucalyptus (1), Macadamia (1), Unknown (16)) (Appendix H). Single sampling plans: one sample from type of honey was selected at random from a lot from local markets or supermarkets and the disposition from private beekeepers in different regions and batches.

3.2 Bacteria

Geobacillus stearothermophilus (DMST 8041) was acquired from the Microbiological Resources Center of Thailand, Institute of Scientific and Technological Research, Thailand. The culture was suspended in Brain Heart Infusion Broth with 20% glycerol and stored at -70^{o}C. Prior to use, the cells were propagated in Nutrient broth at 65^{o}C for 18-24 hours under aerobic condition and they were streaked on Nutrient agar plates to check contamination by incubating at 30^{o}C for 72 hours under aerobic condition.

3.3 Equipment

- Autoclaves (ALP Co., Ltd, CL-40MDVP, Japan) and (JSP Pharmaceutical Manufactory (Thailand) Co.,Ltd ,EMH-16SA, Thailand)
- Lyophilizer (Christ, alpha 1-4, Germany)
- Freezer -80^{o}C (Forma®, 8600, USA)
- Freezer -20^{o}C (Sanyo®, cool safe, Thailand)
- pH meter (Cyberscan, 310, Singapore)
- Hand refractometer (ATAGO, N-3E, 58-90^{o}Brix, Japan)
- HPLC (Agilent technology, HP 1200 with diode array detector and 24 port vacuum extraction manifold assembly, USA)

- Colorimeter (Minolta, Chroma Meter CR 300, Japan)
- Water Activity Meter (AQUA. LAB, 3TE, USA)
- Spectrophotometer (Spectronic 20 genesys TM, 4001/1, USA)
- Waterbath (Memmert, WB14, USA)
- Hotplate stirrer (IKA®, HTS 1003, Germany)
- Pipette controller (Jencons®, Powerpette, England)
- 4 decimal Electronic analytical balance (OHAUS, Explorer, Germany)
- 3 decimal Electronic analytical balance (OHAUS, TS 400D, Germany)
- 1 decimal Electronic analytical balance (OHAUS, CS-200, Germany)
- Refrigerated centrifuge (Hettich Zentrifugen, Universal 32 R, Germany)
- Hot air oven (Memmert, TS8000, USA)
- Incubators (Memmert, BE 500 and BE 600, USA)
- Masticator (IUL®, CE 200, Spain)
- Refrigerator (Mitsubishi®, Tiara, Thailand) and (Whirlpool®, Home Appliances, Thailand)
- Biohazard laminar flow (International scientific supply co.,ltd, BVT-124#269, Thailand)
- Vortex mixer (laboratory& Media Supplies, VTX-3000L, Korea)
-10-100 and 100-1000 µl Variable Volume Micropipettes (Gilson, France) and (Brand®, Germany)
- 50 ml Sterile centrifuge tubes (Corning®, USA)
- 100, 500 and 1000 ml Graduated cylinder (Witag, Germany)
- 1, 5, 10, 20 ml Graduated pipettes (HBG, Germany)
- 500 and 1000 ml Erlenmeyer flasks (Witag, Germany)
- 50, 100, 500 and 1,000 ml glass bottles (Duran®, Germany)
- 90X15 mm Glass Petri dishes (Petriq®, PET 4009015, Germany)
- plastic vernier caliper (Parker®, USA)
- 16 x 125mm (15ml) Glass test tubes (Pyrex, Culture screw cap, USA)
- 1000 ml volumetric Erlenmeyer flask (Pyrex, USA)

- Dispensing spoons
- Aluminum foil (Diamond, USA)
- 200 and 1000 µl Pipet tips (Oxygen, USA)
- Cork borer (Nonaka Rikaki, No. 2, Japan)
- Metal rack
- Microcentrifuge tubes (Hycon, E1004, USA)
- Microtube freezer boxes
- 12x75 mm falcon tubes, round-bottom with snap cap (BD®, USA)
- Latex examination gloves (Siamsempermmed®, Thailand)
- 18 G x 1 ½" (1.2x40 mm) needles (Nipro®, Thailand)
- Timer

3.4 Chemical reagents

- Agar bacteriological; Agar No.1 (Oxoid, England)
- Amitraz 12.5%; $C_{19}H_{23}N_3$ (Eclip, Thailand)
- Beef extract (Lab-scan Co., Ltd, Thailand)
- Bromocresol purple; $C_{21}H_{16}Br_2O_5S$ (Merck, Germany)
- Buffer solution pH 4, 7,10 (Fisher Scientific, UK)
- Calcium chloride dihydrate; $CaCl_2\ 2\ H_2O$ (Carlo Erba, Italy)
- Chlortetracycline; $C_{22}H_{23}ClN_2O_8 \cdot HCl$ (Sigma Chemical Co., C4881-25g, USA) 78% purity (HPLC)
- Deionized water
- D-fructose anhydrous; $C_6H_{12}O_6$ (Merck, Germany)
- D-glucose anhydrous; $C_6H_{12}O_6$ (Merck, Germany)
- Di potassium phosphate; K_2HPO_4 (Ajax Finechem Ltd., New Zealand)
- Ferric chloride; $FeCl_3.\ 6\ H_2O$ (Fisher Scientific, UK)
- Flumetrin (Bayticol 6%); $C_{28}H_{22}Cl_2FNO_3$ (Bayer, Thailand)
- Gelatin (Cartino Gelatin Co., Ltd.,Thailand)
- Glycerol (Merck, Germany)

- Gum Arabic (Jumbo Acacia Co., Ltd, Thailand)
- Hydrochloric acid; HCl (Merck, Germany)
- Isopropanol; $(CH_3)_2$ CHOH (The United Drug (1996) Co., Ltd., Thailand)
- L-cystein hydrochloride; $C_3H_7NO_2S$.HCL anhydrous (Fluka Chemical Company, Switzerland)
- Maltose (BBL™, USA)
- $MgSO_2.7\ H_2O$ (APS finechem, Australia)
- Monopotassium phosphate; KH_2PO_4 (Merck, Germany)
- Naphthalene 99.8%; $C_{10}H_8$ (S.P.B Center, Thailand)
- Olive oil (Italian Trade Commission, Thailand)
- Oxytetracycline dihydrate; $C_{22}H_{24}N_2O_9 \cdot 2H_2O$ (Sigma Chemical Co., O4636-10g, USA) 97% purity (HPLC)
- Peptone from meat (Merck, Germany)
- Skim milk power (Merck, Germany)
- Sodium chloride; NaCl (Merck, Germany)
- Sodium glutamate (World indent business, Thailand)
- Sodium hydroxide; NaOH, AR grade (Merck, Germany)
- Sodium phosphate Na_2HPO_4 (Merck, Germany)
- Soytone; Papic Digest of soybean meal (Hardy diagnostics, USA)
- Starch; $(C_6H_{11}O_5)$ (Merck, Germany)
- Sucrose (BBL™, USA)
- Sulphur 99.5% (The Siam Chemicals Public, Thailand)
- Test sticks of hydrogen peroxide (Quantofix®, Peroxid 25, Germany)
- Tetracycline; $C_{22}H_{24}N_2O_8 \cdot xH_2O$ (Sigma Chemical Co., 87128-25g, USA) 98% purity (HPLC)
- Tryptone; Casein enzyme Hydrolysate, Type-I (HIMEDIA, India)
- Tween 80; $C_{64}H_{124}O_{26}$ (Ajax Finechem Ltd., Newzeland)
- Yeast extract (Merck, Germany)

3.5 Microbiological media

- Brain heart infusion; BHI (Oxoid, England)
- Nutrient agar; NA (Merck, Germany)
- Nutrient broth;NB (Merck, Germany)
- Plate count agar; PCA (Merck, Germany)
- Yeast extract glucose chloramphenicol agar; YGC (Merck, Germany)

3.6 Research designs and methods

Before test, the prepared negative honey sample (longan honey) was kept at 4-8^{o}C in a refrigerator and examined the microbiological, chemical, physical properties. Proper method to eliminate natural inhibines in honey by sterile diluted solution and heated sample was investigated. Test kits were prepared in freeze-dried and normal forms. Detection limits and validation of the test kit with HPLC method was carried out. Shelf-life of antibiotic residues test kit was studied. The test kit was applied for antibiotic screening test on commercial honey samples.

3.6.1 Optimal method for reduction of inhibines in honey

3.6.1.1 Determination of hydroperoxides of honey

The first, the negative honey sample was prepared to eliminate its osmotic or inhibine effect by using 90-50% dilutions with sterile deionized water on pH 5, compared with 100 % concentration honey. The negative honey sample was incubated at 30^{o}C for 5 minutes, 1 hour and 3 hours, 65^{o}C for for 5 minutes, 3 hours, 80^{o}C for 3 hours, 85^{o}C for 5 minutes and 95^{o}C for 5 minutes. H_2O_2 was determined using QUANTOFIX ®Peroxide 25**.** A test stick was dip in the sample for about 1 second, shake off excess liquid and read off at 15 seconds by comparing colour to standard value on the package of test sticks. All assays were repeated in five times for each sample.

3.6.1.2 Effect of pH and concentration of honey on inhibition zone diameter

Media of agar diffusion test: It must be able to support rapid growth of regular clinical isolates, produce clearly-defined zones of inhibition, consist of a standard nutritional formula which is routinely reproducible and, in most laboratories, be free of antibiotic antagonists. In this study, nutrient agar same as research of Patricia *et al.* (2005) was used. pH of the medium was maintained at 7.2-7.4. A standard volume 25 ml of medium was dispensed into petridish and stored between 4-8^{o}C for no longer than 7 days. Prior to use, the plates were stored in an incubator until no surface moisture remains.

Pure culture of micro-organisms was grown on nutrient agar. Five colonies were picked using an inoculation loop into 4 ml of nutrient broth. After incubation at 65^{o}C for 24 hours, the inoculum was prepared by diluting to a 2.51±0.01 optical density at 450 nm. The freshly prepared suspension is shaken. A suspension of the tested microorganism (0.1 ml of 10^{8} cfu/ml) of inoculum was spread on the surface of nutrient agar plate in triplicates with a sterile glass rod. Plates were allowed to stand at room temperature for 15 minutes. Six wells were cut per plate by sterilized cork borer. The well diameter was 5 millimeter. Agar in the well was removed aseptically with sterile needles. One hundred microlitres of sample is placed into each well. Honey solution was prepared under aseptic techniques.

The pH of honey was adjusted in the range of 2.5 to 7.0 to eliminate its osmotic or inhibine effects on microbial assay. Honey solutions were prepared at 10-50% concentrations with sterile deionization water (pH 5). Pure honey was used as control. Plates were incubated at 65^{o}C for 18 hours under aerobic condition. Measurement of zone diameter was done using calipers. The experiment was done with three replicated.

3.6.2 Production of test kit

3.6.2.1 Preparation of media

Each medium was mixed with deionized water and brought volume to 100 ml. Adjusted pH to 8.0±0.2 at 30°C with 4 M NaOH. After completion of sterilization at 121°C for 15 minutes, 0.4 ml of medium was dispensed in micro vial polypropyline with cap and 0.1 ml of the bacteria suspension was added. Ingredients used in microbiological media for preparation of test kit were shown in Table 3.1

Table 3.1 Composition of the media

Media	Carbon source	Nitrogen source	pH buffer	pH indicator	Mineral source	Emulsifier
A	Maltose 10.0%	Gelatin 5.0%, Tryptone 1.0%, Yeast extract 0.5%, Sodium glutamate 2.0%		Bromocresol purple 0.0084%	NaCl 4.0%	
B	Gum arabic 1.0%, Agar 1.5%, Olive oil 1.0%	Peptone 0.30%, Yeast extract 0.1%		Bromocresol purple 0.006%	NaCl 0.5%, $CaCl_2$ 2 H_2O 0.05%, $MgSO_2$.7 H_2O 0.01%, $FeCl_3$. 6 H_2O 0.004%	
C	Sucrose 10.0%	Gelatin 5.0% Sodium glutamate 2.0 %		Bromocresol purple 0.006%		
D	Sucrose 5.0%	Skim milk 10.0%		Bromocresol purple 0.006%		
E	Sucrose 5.0%, Agar 1.5%	Skim milk 10.0%		Bromocresol purple 0.006%		

Table 3.1 Composition of the media (Continue)

Media	Carbon source	Nitrogen source	pH buffer	pH indicator	Mineral source	Emulsifier
F1	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%	K_2HPO_4 0.025%	Bromocresol purple 0.004%	NaCl 0.05%	Tween 80 0.1%
F2	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%	K_2HPO_4 0.025%	Bromocresol purple 0.006%	NaCl 0.05%,	Tween 80 0.1%
F3	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%	K_2HPO_4 0.025%	Bromocresol purple 0.008%	NaCl 0.05%	Tween 80 0.1%
F4	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%	K_2HPO_4 0.025%	Bromocresol purple 0.01%	NaCl 0.05%	Tween 80 0.1%
F5	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%		Bromocresol purple 0.004%	NaCl 0.05%	Tween 80 0.1%
F6	Glucose 0.2%, Starch 0.8%, Agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%		Bromocresol purple 0.006%	NaCl 0.05%	Tween 80 0.1%

Table 3.1 Composition of the media (Continue)

Media	Carbon source	Nitrogen source	pH buffer	pH indicator	Mineral source	Emulsifier
F7	Glucose 0.2%, Starch 0.8%, agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%		Bromocresol purple 0.008%	NaCl 0.05%	Tween 80 0.1%
F8	Glucose 0.2%, Starch 0.8%, agar 1.5%	Beef extract 0.15%, Tryptone 0.6%, Peptone 0.5%, Soytone 0.03%		Bromocresol purple 0.01%	NaCl 0.05%	Tween 80 0.1%

*Media A; Applied media from Reyed (2007)

*Media B; Product Number: 2112677 of BD Bacto^{TM} Peptone

*Media C-E; Media from Reyed (2007)

*Media F1-F8; Applied media from Sinetanu *et al.* (2001)

3.6.2.2. Preparation of bacteria culture

Geobacillus stearothermophilus (DMST 8041) was acquired from the Microbiological Resources Center of Thailand, Institute of Scientific and Technological Research, Thailand. Prior to use, cells were streaked on nutrient agar plates and incubated at 65^{o}C for 24 hours under aerobic condition. After incubation, several colonies were propagated in nutrient broth at 65^{o}C for 72 hours and centrifuged at 2192 *g* for 20 minutes at 25^{o}C, washed three times with sterile normal saline and then incubated at 95^{o}C for 20 minutes (Holt *et al*., 1994). This condition destroyed all vegetative forms of the other known bacteria. The final cells were harvested by cooling centrifugation at 2192 *g* for 20 minutes at 4^{o}C and suspended in normal saline to the concentration of 10^{8}-10^{9} cfu/ml.

3.6.2.3 Preparation of antibiotic standard solutions

Antibiotic standard solution was prepared fresh daily and kept below 4-8^{o}C. They were transfered to 1000 ml volumetric flask and made up to volume using deionized water on pH 5 and mix well. Oxytetracycline, tetracycline and chlortetracycline were individually prepared at 10-1000 µg/kg in spiked positive honey samples.

3.6.2.4 Preparation of acaricide standard solutions

Amitraz (12.5%), flumetrin (Bayticol 6%), naphthalene (99.8%) and sulphur (99.5%) were obtained from Eclip company of Thailand, Bayer Co., Ltd of Thailand, S.P.B Center Co., Ltd of Thailand and The Siam Chemicals Public Co., Ltd. of Thailand, respectively. Solutions of amitraz, flumetrin, naphthalene and sulphur were deionized water at pH 5. The solutions were diluted to the required 3 concentrations of acaricide (For enumeration prepare 10^{-1}, 10^{0} and 10^{1} dilutions of the acaricides at MRL in honey) for spiked positive samples.

3.6.2.5 Preparation of test kit

3.6.2.5.1 Normal test kit

The bacteria were prepared at 10^{8}-10^{9} cfu/ml, after that 0.1 ml of inoculum was added into 0.4 ml of each medium and mixed together by a vortex mixer. Test kits were stored at 4-8^{o}C in darkness until testing.

Figure 3.1 0.1 ml of bacteria and mixture 0.1 ml of bacteria and 0.4 ml media

3.6.2.5.2 Freeze-dried test kit

The bacteria were prepared at 10^8-10^9 cfu/ml, after that 0.1 ml of inoculum was added into 0.4 ml of medium and mixed together by a vortex mixer. Test kits were separated into two groups. The first group was stored at -20°C for 18 hours and the second group was dropped in liquid nitrogen for 5-10 minutes, after that they were taken in a lyophilizer (Christ, alpha 1-4, Germany) for 24 hours. Test kits were stored at 4-8°C in darkness until testing.

3.6.3 Preparation to test the test kit

Negative controls were prepared using negative honey sample diluted 90-60% in diluent (Horwitz, 2000). Diluent A (sterile deionized water on pH 5), diluent B (0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 on pH 5), diluent C (0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 on pH 6), diluent D (0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 on pH 7), diluent E (0.004 M KH_2PO_4 with 0.1 M K_2HPO_4 on pH 6) and diluent F (0.004 M KH_2PO_4 with 0.1 M K_2HPO_4 on pH 7) and 0.1 ml of 10-40% concentration of honey were added at 30, 65, 85 and 95°C for 5 minutes.

Positive controls were prepared using negative honey sample diluted 90-60 % in diluent A (sterile deionized water on pH 5), by adding 10-1000 µg/kg antibiotics in 10-40% concentration of honey at 30, 65, 85 and 95°C for 5 minutes. Tetracycline, Oxytetracycline and Chlortetracycline were used as standard antibiotics (Sigma Chemical Co., USA).

Negative and positive controls were incubated at 65°C for 120-180 minutes. The test was read negative or positive by the change of colour. In the absence of antimicrobial substances, the whole solid medium turned into total yellow colour. The beginning time to read positive controls or samples was when the negative controls turned into yellow colour completely. The medium remained purple in the presence of sufficient concentrations of antibiotics. At intermediate concentrations of antibiotic, the solid medium turned partly yellow. The results were read positive when the yellow colour

was formed in the bottom three-fourth parts of the ampoule. The experiment was carried out using a complete randomized design (CRD) with 5 replications. Comparison of means was conducted using analysis of variance (ANOVA) to select the best screening test kit. The best screening test kit was results of the negative controls turned into yellow colour completely, the positive samples were easy to separate colour (yellow and purple) and minimum detection limit was very low.

3.6.4 Validation of test kit with HPLC technique

The result obtained from the best screening test kit using optimal formula and test condition was validated and understanded how to quantitatively analyze the performance with HPLC technique (Pena *et al*., 2005)**.** Analysis of tests was validated based on sensitivity, specificity and accuracy of the test (Smith, 2006).

The method was studied 100 blank samples and 100 spiking blank samples at five concentration levels (10, 50, 100, 500 and 1,000 µg/kg). Repeatability was performed using 20 replicates for each concentration level in one day with two different batches.

The detection limit values of several classes of antibiotics (oxytetracycline, tetracycline and chlortetracycline) were studied with spiking blank samples at 5, 10,100 µg/kg with 5 replication between screening test kit and HPLC technique .

3.6.5 Effect of storage time on effectiveness of test kit

The test kit with optimal components for the microbial assay was stored at 4-8^{o}C. Effectiveness of the test kit was evaluated during the storage from 1 to 270 days. The assay was conducted using diluted honey without antibiotic and the diluted honey with antibiotic 10-100 µg/kg (tetracycline, chlortetracycline and oxytetracycline) in optimal diluted solution. The experiment was conducted using a complete randomized design (CRD) with 5 replication. ANOVA and LSD analysis were conducted with the data at 95% confidential level in order to compare mean values.

3.6.6 Application of test kit for acaricide and antibiotic residues

3.6.6.1 Determination of acaricides in honey

The method was studied spiking blank samples at three concentration levels (For enumeration prepare 10^{-1}, 10^{0} and 10^{1} dilutions of the acaricides at MRL in honey). Maximal residue limits of amitraz, flumetrin, nephthalene and sulphur were 200, 5, 10, 10 µg/kg, replications. Repeatability was performed using 10 replicates for each concentration level of acaricides in one day with two different batches by test kit.

3.6.6.2 Determination of antibiotic in honey

Honey samples of different geographical origins from the north of Thailand were obtained from private beekeepers in different regions of Thailand (76 samples) and local markets or supermarkets (44 samples). Total 120 honey samples were examined for the physical properties as follows: color quality by using colorimeter (Minolta, Chroma Meter CR 300, Japan), pH by using pH meter (Cyberscan, model 310, Singapore), total soluble solid content by using hand refractometer (ATAGO, model N-3E, Japan), and water activity (a_w) by using water activity analyzer (AQUA. LAB, model Series 3TE, USA). All tests were done in triplicate except color quality that was done in ten replicates. The prevalence of tetracycline group residues in 120 honey samples by screening test kit was studied. The honey samples were collected during June-August 2010. The study of tetracycline group residues had been limited to 30 samples by HPLC technique, followed by the sampling of 30 samples to estimate percentage by Win Episcope (Thrusfield *et al*., 2001).

3.6.7 Determination of antibiotic residues by HPLC technique

The sample of honey (10-15 g) was weighed into a polypropylene tube and dissolved in 50 ml of 0.1 M Na_2 EDTA-McIlvaine buffer (pH 4.0). The sample solution was shaken for 5 min and centrifuged at 2,850 g for 15 minutes. After filtration, it was loaded on C 18 Clean-up (VertiPak ^{TM}) 6 cm^3 (500 mg) cartridge previously conditioned

with 5 ml of methanol and 5 ml of deionized water. The cartridge containing the sample was then washed with 5 ml of methanol. Then, the tetracyclines were eluted with 5 ml of oxalic acid (1 M)/acetronitrile (80/20). The final eluate was filtered through a 0.45 µm cellulose acetate membrane filter and stirred in vortex before injecting into the HPLC system. The tetracycline residue separation was carried out on a HPLC system (Agilent, HP 1200, USA). HPLC analyses of tetracycline group residues were applied according to Geertsen and Pedersen (2009) by Euroresidue Conferences, Euroresidue IV, 2000 with a Diode Array Detector and Zorbax Eclipse Separation Column (150 x 4.6 mm, 5 µm) at a light wavelength of 356 nm with C 18 Guard column. The mobile phase was acetonitrile: methanol: oxalic acid 0.01M (10: 10: 80) at flowrate 1.0 ml/minutes, injection volume 100 µl, column temperature 30^{o}C. The mean recovery was 88.79% for tetracycline, 95.46% for oxytetracycline and 81.17% for chlortetracycline, all at 10 µg/kg. The calibration graphs were constructed with standard solution concentrations ranging from 13-74 µg/kg. The linearities were evaluated by linear regression analysis, which was calculated by least square regression method. All the solvents used (acetonitrile, methanol and ethyl acetate) were of HPLC grade. Other reagents were of analytical grade and purchased from Merck Ltd. Water used was of HPLC grade.

3.6.8 Statistical evaluation

All results when possible were statistically analyzed by analysis of variance (Excel® 2003). If a significant main effect was detected, the means are followed by ± *x*, indicate whether *x* refers to the standard deviation. The predetermined acceptable level of probability was 5% ($p \leq 0.05$) for all comparisons (e.g., *t*-tests, one-way analysis of variance).